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„Nature's pharmacy – how *Azteca* sp. ants keep their colony clean, using the host plant *Cecropia* sp."

verfasst von / submitted by

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1 Acknowledgement

I would like to take this opportunity to thank Veronika Mayer, who has opened all doors and gates for me and has allowed me to carry out really exciting experiments.

Furthermore, my thanks go to the institute of science and technology, especially to the whole group of Sylvia Cremer, who helped me to carry out the experiments and provided their equipment. Special thanks go to Barbara Milutinovic from the Cremer group, who mainly struggled with me, patiently answering all of my questions, taking a lot of her time and supporting me with great zeal. She was also always a great help next to Veronika in evaluating the results.

In addition, I would like to thank my good friend Johanna Trupke, who has given me a lot of input and has proofread my thesis.

And last but not least, I would like to thank my parents, who always support me without exception.

1.1 Further

Earlier this year (March 19th-22nd, 2019) the project was presented at the Central European Meeting of the International Union of the Study of Social Insects (IUSSI) at the Institute of Science and Technology Austria (IST Austria) in Klosterneuburg.

Do antimicrobials play a role in the symbiosis between *Azteca* ants and *Cecropia* plants?

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Introduction & Questions

The *Cecropia–Azteca* association is one of the most ubiquitous ant-plant mutualisms in Neotropical ecosystems. The plants provide ants with nesting space in their hollow stem internodes (Fig. 1) as well as glycogen-rich food bodies. In return for shelter and food, *Azteca* react with impressive aggression towards herbivores and overgrowing plants. Recent studies have shown that *Azteca* regularly make patches comprised of parenchyma, dead nest mates, and other debris. Surprisingly, these patches are also found next to the brood.
In the current study we are investigating, how the larvae are protected against microbial attack from the nearby patches. To this purpose we tested the antimicrobial activity of the resident *Azteca* ants, the whole patch, ant-made carton walls used for brood deposition, and *Cecropia* leaves.

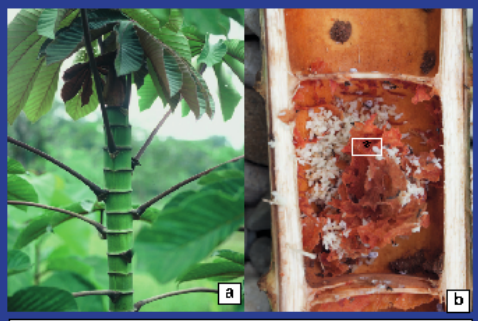
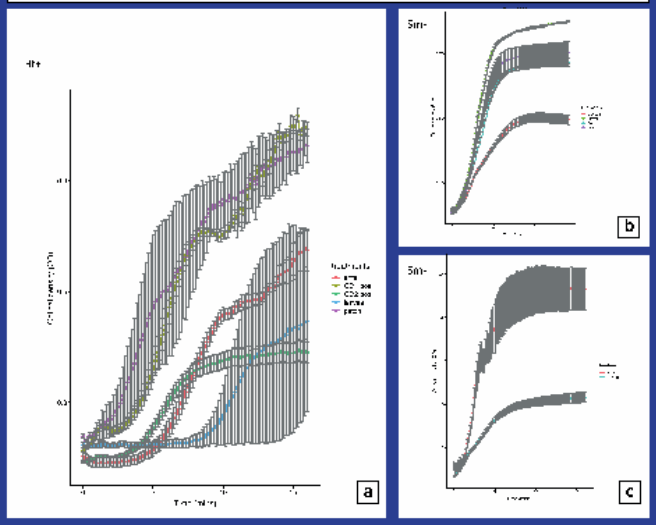


Figure 1: A stem of *Cecropia obtusifolia* from the outside (a) and opend (b). *Azteca* ants nest in the hollow internodes and deposit the larvae on structures made from the parenchyme of the inner internode wall (carton).



Results

In the plate reader assays the leaves of *Cecropia obtusifolia* showed an inhibitory effect on *Bacillus thuringiensis* (*Bt*, gram+) (Fig. 2a). Extracts from patch and resident ants, however, did not inhibit bacterial growth, extract from patch samples even enhanced growth (Fig. 2a).

An inhibitory effect on *Serratia marcescens* (*Sm*, gram-) could not be found (Fig. 2b, c).

The effect of carton extract on bacterial growth was tested on LB medium. Carton extract had an inhibitory effect on *Bt* (gram +) (Fig. 3), but not on *Sm* (gram-) (Fig. 4).

Figure 2: Plate reader assays. (a) ***Bacillus thuringiensis*** **(gram +) with extract of *Azteca* ants, *Cecropia* leaves and patch,** CO2 pos and + the positive control against the *Cecropia* samples, CO2 pos is the positive control against the *Azteca* ant samples. (b) ***Serratia marcescens*** **(gram -) with extract of *Cecropia* leaves and patch,** CO pos and is the positive control against the *Cecropia* samples. (c) ***Serratia marcescens*** **(gram -) with extract of *Azteca* ants.** Curves represent mean and standard error for each of the biological replicates. CO pos is the positive control against the *Azteca* ant samples.

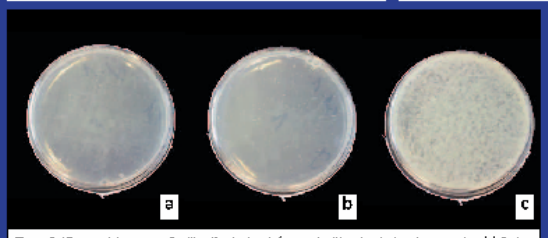


Figure 3: LB agar plate assays. *Bacillus thuringiensis* (gram +) with extract of carton samples. (a) Carton sample CO. (b) Carton sample C7. Only a few single cell colonies grew on these agar plates. (c) Control. Nearly the whole control plate was overgrown.

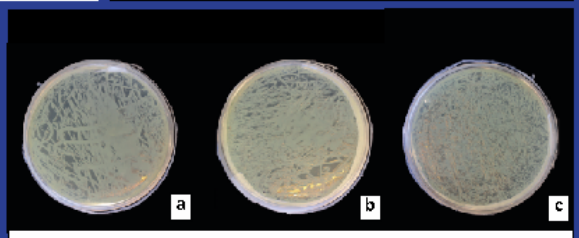


Figure 4: LB agar plate assays. *Serratia marcescens* (gram -) with extract of carton samples. (a) Carton sample C1. (b) Carton sample C7. (c) Control. All plates with extract and control were almost completely overgrown.

Conclusion

- We could find an inhibitory effect of gram+ bacteria with leaf and carton extracts but no inhibitory effect on gram – bacteria. This indicates that the host plant itself provides secondary metabolites which are able to inhibit the growth of gram+ bacteria. This could explain, why carton is always crowded with larvae and pupae. Carton seems not only to serve for brood shelter but also help to protect the larvae and pupae against certain pathogens.
- Extract of ants did not have any inhibitory effect. This could be due to the fact that we tested silica gel dried samples because we had no access to fresh material. Antimicrobial substances may be degraded or inactivated by the drying.

Future work

- All tests will be repeated with fresh material
- Additionally we will test wether leaves, carton, ants or patch can inhibit the growth of entomopathogenic fungi.

2 Abstract

The *Cecropia–Azteca* association is one of the most ubiquitous ant-plant mutualisms in Neotropical ecosystems. The plants provide ants with nesting space in their hollow stem internodes, as well as glycogen-rich food bodies for the ants to feed on. In return for shelter and food, *Azteca* ants protect the plant against herbivores and overgrowing vines.

Recent studies have shown that *Azteca* ants regularly make so-called *patches*, comprised of organic matter (parenchyma, dead nestmates, and other debris). On these patches also bacterivorous nematodes and melanised, slow-growing fungi of *Chaetothyriales* (Ascomycetes) are found. The fungus is then fed to the larvae. Such patches are found in all compartments of the nest and as the larvae are fed with it, also next to the brood. Due to the warm and humid conditions in the tropics, the colony is under continuous threat from fungal or bacterial pathogens infesting these patches and hence, the nearby brood.

In the current study it was investigated, how the resident ants minimize microbial attack. Using microplate and agar plate bioassays with two insect pathogenic bacteria and one insect pathogenic fungus, we discovered that especially the leaves of the plant had an inhibitory effect of the growth of gram-positive *Bacillus thuringiensis (Bt.)* but not on gram-negative *Serratia marcescens (Sm.)*. Observing the growth of *Bt.* on LB agar plates in combination with ant made carton where ant larvae are usually placed, I could show, that also inhibition of bacterial growth occured. This observation shows that ants use plant materials with antimicrobial activity to prepare the nest area where they store their brood.

In fungus-fungus competition assays against an entomopathogenic *Metarhizium* sp. (Ascomycota, Hypocreales) I could further show, that pure cultures of ant associated Chaetothyriales could block the fast growing hyphae of *M. brunneum*.

In conclusion, the present work paves the way for future experiments to unravel the role of the individual components of the *Azteca* colony with respect to antimicrobial activity. This could potentially also lead to the discovery of new drugs.

3 Introduction

The term mutualism describes a system between different species from which all parties benefit (Gutiérrez-Valencia et al., 2017). The mutualism between *Cecropia* sp. (Urticaceae) plants (Fig. 1A) and *Azteca* sp. (Dolichoderinae) ants is one of the most pervasive interactions in Neotropical ecosystems. The naturally hollow stem internodes of the trees is used by the ants for nesting (Marting et al., 2018). The plant also provides phyto-glycogen-rich food bodies, the Müllerian bodies (Fig.1B), on which the ants feed. They are produced on the trichilium, a dense mat of trichomes at the base of the leaf petiole (Bischof et al., 2013). In return for shelter and food, *Azteca* ants protect the trees against herbivores and remove encroaching vegetation, like vines, with impressive aggression (Schupp, 1986; Agrawal & Dubin-Thaler, 1999). *Azteca* ants fabricate cardboard like structures ("carton") from by masticating plant fibers, e.g. the parenchyma from the inner domatia wall (Fig. 1C) and it is used to structure the domatia of the plant in different compartments (Nepel et al., 2014).

Recent studies have shown that *Azteca* regularly make patches comprised of parenchyma, dead nest mates, and other debris inside domatia (Nepel et al., 2016; Mayer et al., 2018). These patches also help exchanging nutrients, especially nitrogen, between the plant and the ants (Sagers et al., 2000). Surprisingly, these patches are also found next to the brood. However, it is unusual that ants store patches with organic waste next to the brood. This imposes the question whether *Azteca* ants can produce specific antimicrobial or antifungal substances in their metapleural glands (myrmicacin), such as leafcutter ants (Ortius-Lechner et al., 2000) or *Lasius* ants which transmit antimicrobial venom from their venom glands to enhance the resistence of brood against diseases (Tragust et al., 2013). Futher studies indicate that this also applies to *Acromyrmex* and *Polyhachis* ants (Tranter et al., 2014).

Moreover, fungi have long been known to be part in plant-ant mutualisms (Defossez et al., 2009). These fungi have only recently been described and belong to the order *Chaetothyriales* (Ascomycota), a slow growing group of "black yeasts" with melanized hyphae. It has been observed that they are fed to the larvae (Blatrix et al., 2012). Few hyphal fragments are brought from the mother colony to the daughter colony, by the young new colony founding queens (Mayer et al., 2018). The fungi were observed to grow into the living plant tissue. It can be assumed that the fungal mutualists are most likely not pathogenic to their host (Defossez et al., 2009).

Another possibility to enhance nest hygiene was described by Haeder et al. (2009). They were able to isolate an antifungal compound (candicidin) which is produced by a *Streptomyces* strain (Actinobacteria) associated with leaf-cutter ants (Haeder et al., 2009).

Furthermore, the leaf extract of *Cecropia* was reported to be used against as an anti-inflammatory (Pérez-Guerrero et al., 2001) drug. These findings lead to the question if it is also used by the ants for their nest hygiene. In this study, we used different colonies of A*zteca* ants and plant parts of *Cecropia* to answer various questions:

(i) Are Azteca ants producing antimicrobial or antifungal substances to keep their brood clean, even though feces, dead ants and other waste are stored next to the brood?

(ii) Can the ants use the medicinal ingredients of the plant while building the carton to keep their brood clean?

(iii) What is the role of the fungus? Do these "black yeasts" produce compounds to control growth of possible pathogens in the domatia?

Figure 1. The *Azteca-Cecropia* mutualism. **(A)** A view on the stem and the apical part of a *Cecropia obtusifolia*. **(B)** Müllerian food bodies on a trichilium. **(C)** A cross-section of the stem shows carton galleries inside the naturally hollow internodes. This photo also shows the brood in these carton galleries and workers of *Azteca constructor*. Photo credit: A, C Veronika Mayer, B Klaus Kaltenbrunner.

4 Material and Methods

4.1 Analysed Samples - Preparation

All buffer, media, solutions and agar plates were prepared by IST Austria media kitchen – find recipes in the "Appendix"

4.1.1 Colonies

The experiments were done with dried and fresh material of leaves, carton, patch, larvae and worker ants. All samples were collected in Costa Rica by Veronika Mayer in a 5 km radius around the Field Station La Gamba, Costa Rica (8° 42’ 03’’ N, 83° 12’ 06’’ W). Sampling

and sample export was under the permission from SINAC (Sistema Nacional de Areas de Conservación de Costa Rica of the Ministry of Environment and Energy - MINAE) (No. INV-ACOSA-013-18). From 3 *Azteca* colonies (including *Cecropia* leaves, patch) and silica gel dried before they were brought to Vienna. Additionally, workers of 3 more colonies were silica gel dried and transported to Vienna. 8 more colonies were collected in Costa Rica and imported alive by Veronika Mayer in April 2019.

Two different species of ants were collected, *Azteca alfari* (Fig. 2A) and *Azteca constructor* (Fig. 2B).

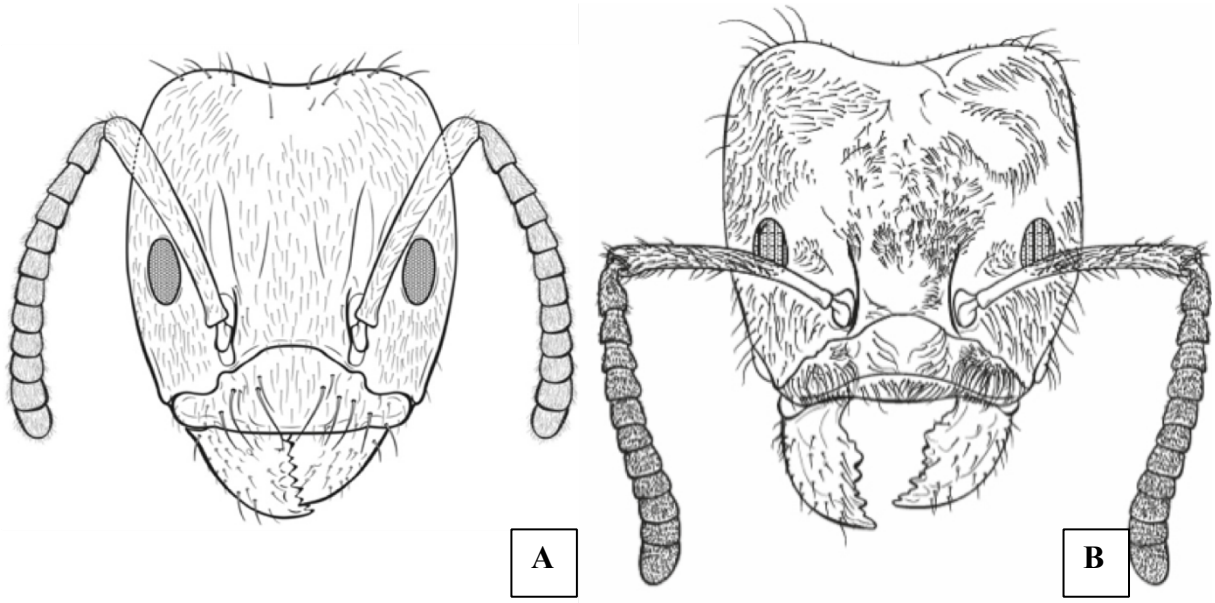


Figure 2. The two collected *Azteca* species **(A)** *Azteca alfari* **(B)** *Azteca constructor*. Both images were made by Katharina Krizan, using Photoshop and Adobe illustrator.

4.1.2 Plant Samples

The needed amount of dried (25mg) and fresh (50mg) patch and leaves was put into 1.5mL Eppendorf-tube. To homogenize the samples, beads (1 ceramic bead - 2.8mm; 325g, 5 Zirconia beads - 1mm, a spoon full of glass beads - acid washed; 425-600µm) were added to each tube. First, the fresh samples were frozen using liquid nitrogen, then all samples were transferred in equal amounts to the pre-cooled sample holders and crushed in the TissueLyser (II; Qiagen) for 2x2 minutes at 30Hz. After the addition of water (dried samples: 300µL, fresh samples: 600µL) lysis was repeated. The tubes were centrifuged for 5 minutes at 3500g (=rcf), at 4°C (Centrifuge 5424R; Eppendorf). Afterwards, the supernatant was transferred into new tubes and the homogenate was ready to be transferred to the 96-well plate (TPP tissue culture plates, 96 well plate, 0,34cm^2, item number: 92696, greiner bio one).

4.1.3 Ant Samples

This protocol was modified after the protocol of Konrad et al. (2012). From each colony workers of *Azteca alfari* or *A. constructor* (5 dried ants, 30 fresh ants to increase the concentration) were transferred into 2.5mL Eppendorf-tubes. Fresh ants were frozen with liquid nitrogen and subsequently PBS buffer (dried ants: 50µL, living ants: 300µL) was added. These samples were further processed as the plant samples (above).

4.1.4 Bacteria

Bacillus thuringiensis (gram-positive) (407 strain) and *Serratia marcescens* (gram-negative) (303 strain) (strains were cultivated at IST Austria) were plated from the long-term storage (-80°C) on LB agar plates and incubated overnight at 30°C. For the experiment, 5 single colonies were inoculated into 10mL of liquid LB media each. These were thoroughly resuspended and incubated at 30°C and shaking at 180rpm. The aim was to test for inhibitory substances while the bacteria are still in the log phase because at this stage they are actively growing and metabolizing. Therefore, it was necessary to measure the optical density (OD) of the suspension regularly (every 20 minutes after the first 4 hours), which should be similar across all experiments and for all bacteria strains, ideally at an OD between 0.2-0.5. The suspension was transferred to a 50mL Falcon© tube and placed on ice to stop bacterial growth.

4.1.5 Fungi

To investigate if the fungus in this mutualism also plays a role in nest hygiene through bioactive substances, I did experiments with two isolates from Chaetothyriales growing in patches of two ant-plant symbioses. One pure fungus culture was from an *Azteca xanthochroa* colony inhabiting *C. insignis* (Cec 13 = ChaeD-CR-3 OTU1, GenBank accession KX120978) and one from *Pseudomyrmex* sp. inhabiting *Triplaris melaenodendron* (GenBank accession KX822551).

For fungus-fungus competition experiments the insect pathogenic *Metarhizium* spp. was used (Roberts & St. Leger, 2004). Spores from a common *Metarhizium* strain (J, KVL 13-14 the culture collection of the Department of Plant and Environmental Sciences, University of Copenhagen, Denmark) (Steinwender et al., 2014) were collected with the following procedure:8ml of 0.05% Triton X solution were pipetted on the plate with a sporulating fungus. All spores were gently scraped off with a glass spreader (4mm diameter glass rod with polished ends, 130mm long handles and 50mm long spreader segments, 120 deg. bend,

Sigma-Aldrich) and transferred to a 15ml Falcon© tube. The solution was centrifuged for 5 minutes at 3000g, the supernatant was carefully discarded, to avoid losing spores from the pellet. The pellet was washed twice by adding 8ml of 0.05% Triton X and mixing well by gently inverting the tube or vortexing until the pellet dissolved. Next, the solution was centrifuged again, and supernatant was poured off carefully. Finally, the pellet was resuspended in 3ml of 0.05% Triton X.

The spores were counted with an automated cell counter (Cellometer® Auto M10 Cell Counter, Nexcelom Bioscience) in a 1:1000 solution. For that, 10µL of stock spore solution were diluted with 990µL 0.05% Triton X. Again, 10µL were taken from the resulting solution and diluted with 90µL 0.05% Triton X. That solution was serially diluted to a $1x10^6$ solution, using the following calculation.

$$\frac{required\ concentration\ \times\ required\ volume}{measured\ concentration}$$

4.2 Experiments

4.2.1 Bacterial inhibition assay – plate reader

To test the inhibitory potential of the homogenized extracts from the colonies (patch, *Cecropia* leaves, worker ants) on bacterial growth, antimicrobial assays (modified from Milutinović, et al., 2015) were performed. We also did this experiment in our first test runs with "fungal water". For this purpose, the fungi (Cec13 & Tri8a) grown on agar plates were covered with distilled water. Based on the assumption that these fungi produce antimicrobial substances and release them into the water, the samples could stand for 2 weeks before the water was taken off with a pipette and also tested with the plate reader assays. However, this experiment did not work.

The samples were mixed with either gram-positive (*Sm.*) or gram-negative (*Bt.*) bacteria. As a positive control (CO1/2) the samples were mixed with either gram-positive or gram-negative bacteria without growth medium (replaced with PBS or H_20). As a negative control (COneg), no bacteria were added. The 96 well plates (TPP tissue culture plates, 96 well plate, 0,34cm^2, item number: 92696, greiner bio one) were prepared on ice. The total volume in each well was 70µl:

Patch	Leaves	Ants	Positive control	Negative control
50µl homogenate + 20µl bacteria (*Bt/Sm*)	50µl homogenate + 20µl bacteria (*Bt/Sm*)	50µl homogenate + 20µl bacteria (*Bt/Sm*)	Plant samples: 50µl bacteria (*Bt/Sm*) + 20µl H_2O Ant samples: 50µl bacteria (*Bt/Sm*) + 20µl PBS	Plant samples: 50µl LB media + 20µl H_2O Ant samples: 50µl LB media + 20µl PBS

To analyze bacterial growth with the different extracts and to find possible inhibitory effects optical density changes were measured with SpectraMax (M2) over 16 hours, at 30°C at 10-minute intervals. For each sample (for biological replicates refer to the table below) at least three technical replicates were measured.

	Ants	Leaves	Patch
Dried Samples	n=6	n=3	n=3
Fresh Samples	n=8	n=8	n=8

4.2.1.1 Processing of plate reader data

All data from the plate reader were processed with RStudio (1.1.463, © 2009-2016 RStudio, Inc). First, growth curves were plotted for each technical replicate. All plots were generated with the R package ggplot2 (Wickham, 2011). Subsequently, the mean and standard error from all technical replicates from the same biological replicate was calculated (Wickham, 2011). Then, the mean and standard error was calculated for all biological replicates of the same sample type and combined into a curve and plotted with their standard error. The same processing was applied to the controls. The R package GrowthCurver (default parameters) (Sprouffske & Wagner, 2016) was used to fit logistic curves to the averaged technical replicates and to extract the growth rates (r) for each biological replicate and the controls.

4.2.2 Bacterial inhibition assay – LB agar plates

Samples of carton and ant larvae had to be tested differently, since the medium turned red/brown (carton) or rather blurry (larvae), so the optical density could not be measured. The samples were prepared as described above (carton was treated like the plant samples, refer to 1.1.2; larvae: was treated like the ant samples, refer to 1.1.3). 50µL supernatant were incubated at room temperature for 2 minutes with 50µL bacteria and pipetted on LB agar plates. The suspension was spread evenly on the plates by shaking these with 5 glass beads (5mmØ). Beads were removed, the plates were sealed with parafilm and placed in the incubator overnight, for 12 hours, at 30°C, allowing for sufficient single colonies to grow if they are not inhibited by the carton extract.

The control for the carton samples consist of 50µL bacteria and 50µL water, while in the controls for the larvae, the water is replaced by PBS. All plates were photographed the next day with a Canon EOS 100D camera and a Canon EFS (18-135mm ISUSM) objective (blend: 11, focal length: 59mm, ISO: 100).

4.2.3 Fungi – contact inhibition assay

For the first series of competition assays 10µL of the *Metarhizium* sp. spore solution were pipetted on SDA plates. As domatia fungi hardly produce spores a 5mmØ mycelium piece of CR13 and Tri8A from a 2% SDA plate was cut out with a metal cork drill and placed opposite to the *Metarhizium* sp. spores. The cut outs were isolates from ant-cultivated fungi in *Cecropia* plants. It was not possible to collect spores from these fungi, because they did not sporulate under controlled laboratory conditions. Three technical replicates per fungus were performed.

For the second treatment 2x 10µL of the spore solution were pipetted on opposite sides of the SDA plate.

The third series of competition assay 2x 5mmØ cut outs of the ant-cultivated fungi were plated on opposite sides of the SDA plate.

The plates were sealed with parafilm and kept at room temperature upside down to avoid condensed water on the agar. The plates were documented on a weekly basis over 9 weeks until the fungi ran out of nutrients and died. Pictures were taken with a Mamiya Leaf Credo 80 reproduction camera equipped with a 120mm Macro at the University of Vienna, Department of Botany and Biodiversity Research.

5 Results

The *Azteca* ants build their colonies inside the hollow stem of a living *Cercopia* tree. Within these colonies, ants have to protect themselves but above all their brood from pathogenic microbes and fungi. However, it is still very little known how these ants are able to keep their colonies clean from bacteria and other pathogens. To identify the source of the microbial and fungal growth inhibitors within the colonies, the individual components (the ants themselves, the plants, the leaves, the brood and fungi) were tested in different experiments.

5.1 Bacteria inhibition assay – plate reader

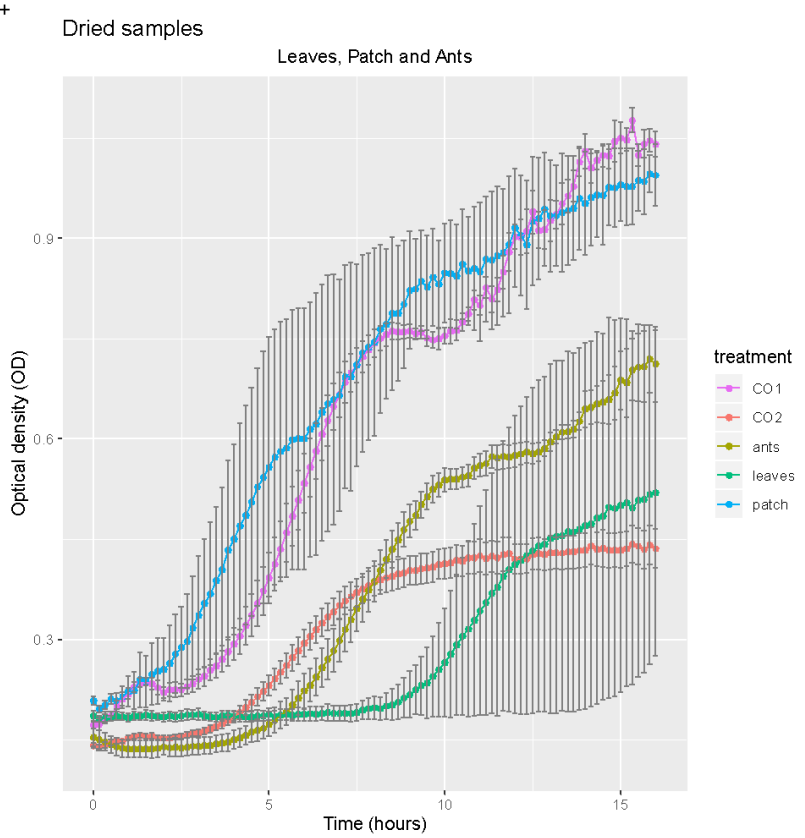
Different parts of the plant (leaves, patch and parenchyma), as well as the ants themselves (worker and larvae), were tested for their ability to inhibit bacterial growth.

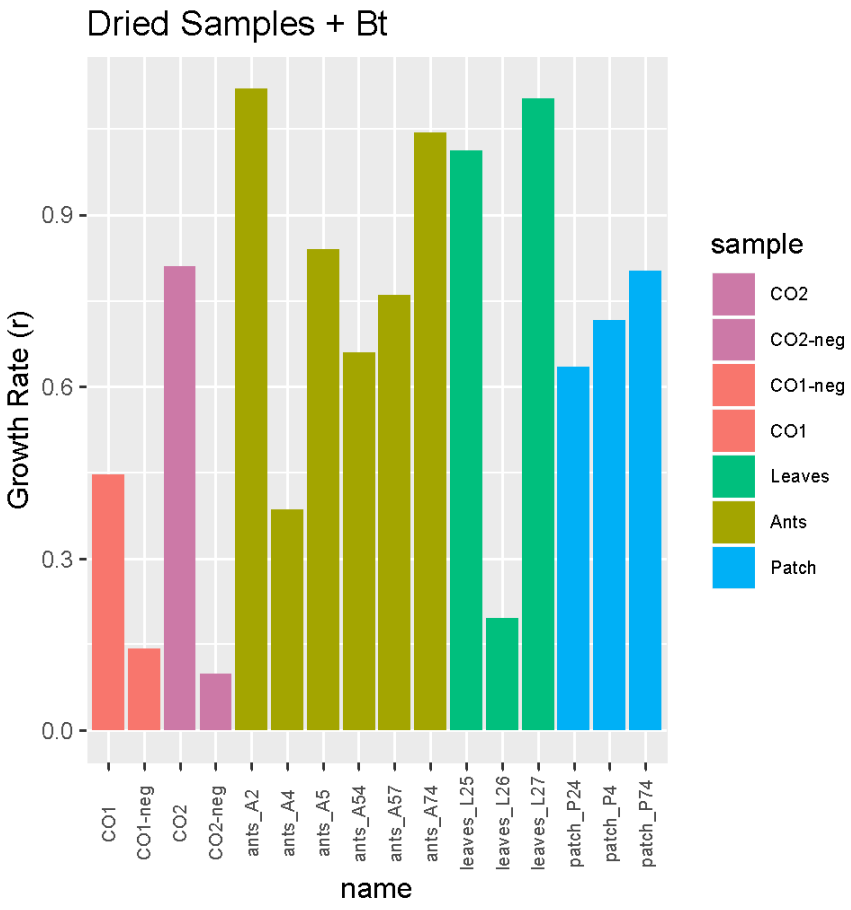
In the first experiment, using the dried samples of three *Azteca* colonies and their respective host plant, the leaves of *Cecropia obtusifolia* had an inhibitory effect on the growth of *Bacillus thuringiensis.* in comparison to the control (CO1 – containing *Bt*) (Fig. 3A). One of the leaf extracts inhibited *Bt.* growth considerably (Fig. 3A second panel). Extracts from the patch, however, did not inhibit bacterial growth, it even enhanced growth (Fig. 3A). Only a mild inhibition could be achieved by the some of the ant samples (Fig. 3A). By comparing the growth rates (Fig. 3A second panel), one can see that the individual biological replicates vary extremely, some of them resulted in an even higher growth rate than the positive control, thereby also explaining the large standard error seen in the averaged growth curves.

In contrast, *Serratia marcescens* (Sm) had a higher final optical density and higher growth rates in combination with the extracts of the different samples (*Cecropia* leaves, patch and *Azteca* ants) than the two positive controls (Fig. 3B). CO1 as the positive control for the plant samples and CO2 as the positive control for the ant samples. In this part of the experiment it could be shown that the different samples had no detectable influence on the growth of gram-negative bacteria (Fig. 3B).

A

Bt+


Dried samples
Leaves, Patch and Ants
Optical density (OD)
0.9
0.6
0.3
0
5
10
15
Time (hours)
treatment
CO1
CO2
ants
leaves
patch


Dried Samples + Bt
Growth Rate (r)
0.9
0.6
0.3
0.0
CO1
CO1-neg
CO2
CO2-neg
ants_A2
ants_A4
ants_A5
ants_A54
ants_A57
ants_A74
leaves_L25
leaves_L26
leaves_L27
patch_P24
patch_P4
patch_P74
name
sample
CO2
CO2-neg
CO1-neg
CO1
Leaves
Ants
Patch

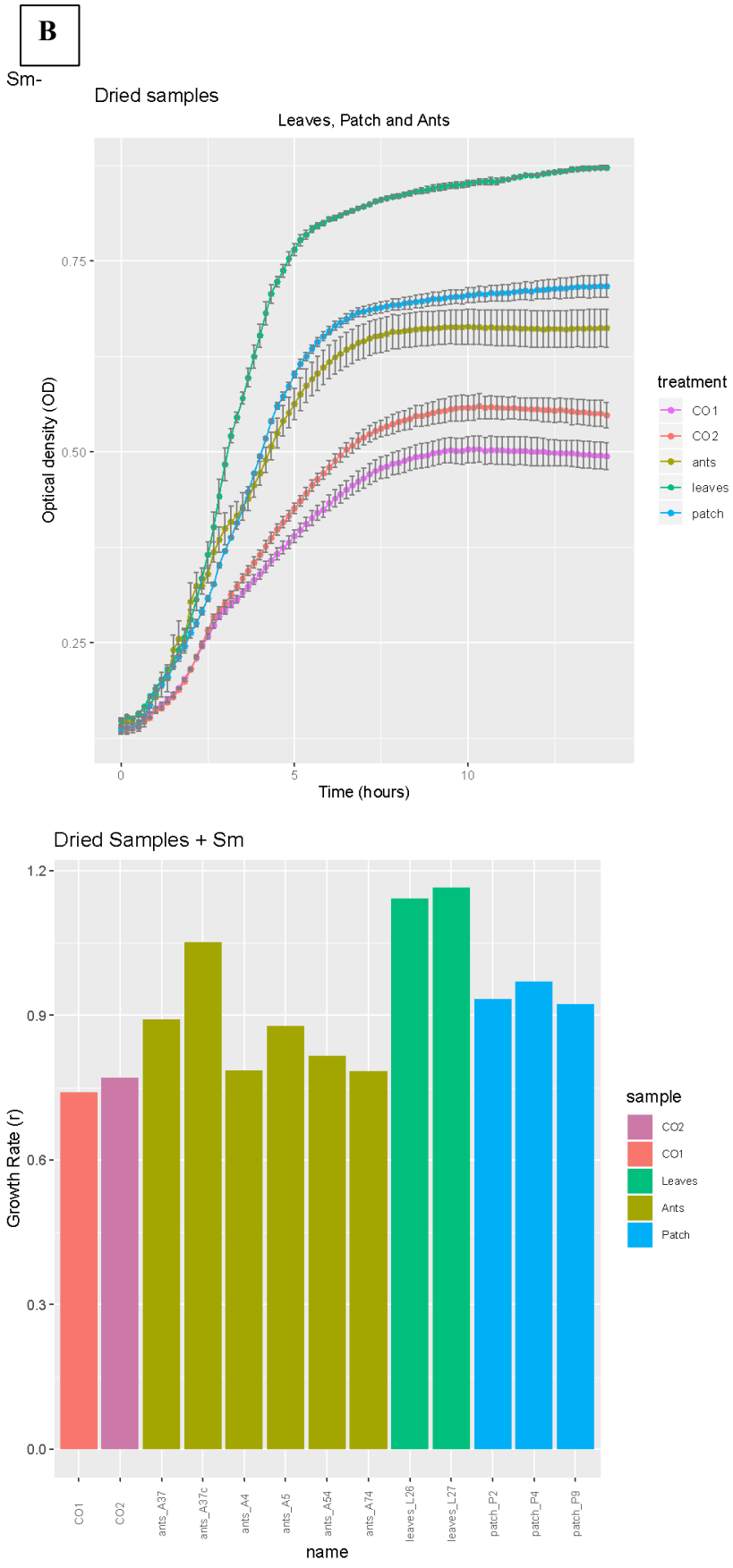


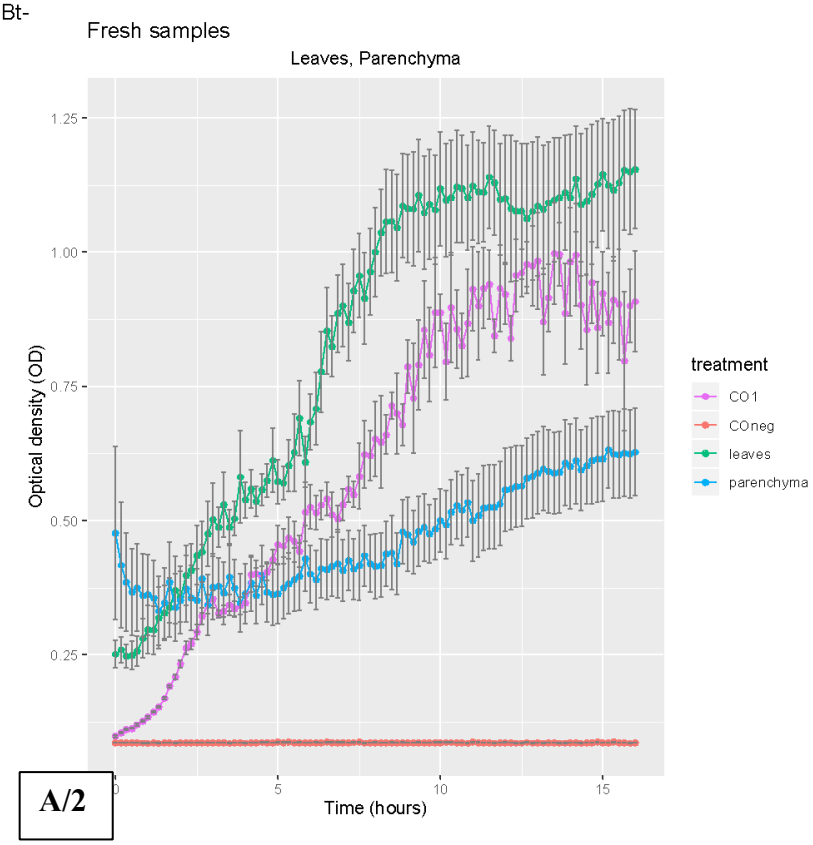
Figure 3. First panels: Mean and standard error for all biological replicates of dried samples, *Cecropia* leaves, patch and *Azteca* ants. Second panels: Growth rate for the individual biological replicates. CO1: positive control for all the *Cecropia* plant samples; CO2: positive control for the *Azteca* ant samples. **(A)** Shows the growth and

growth rates of *Bt*. with the different extracts and controls. **(B)** Shows the increase of *Sm.* and the growth rates with the different extracts and controls.

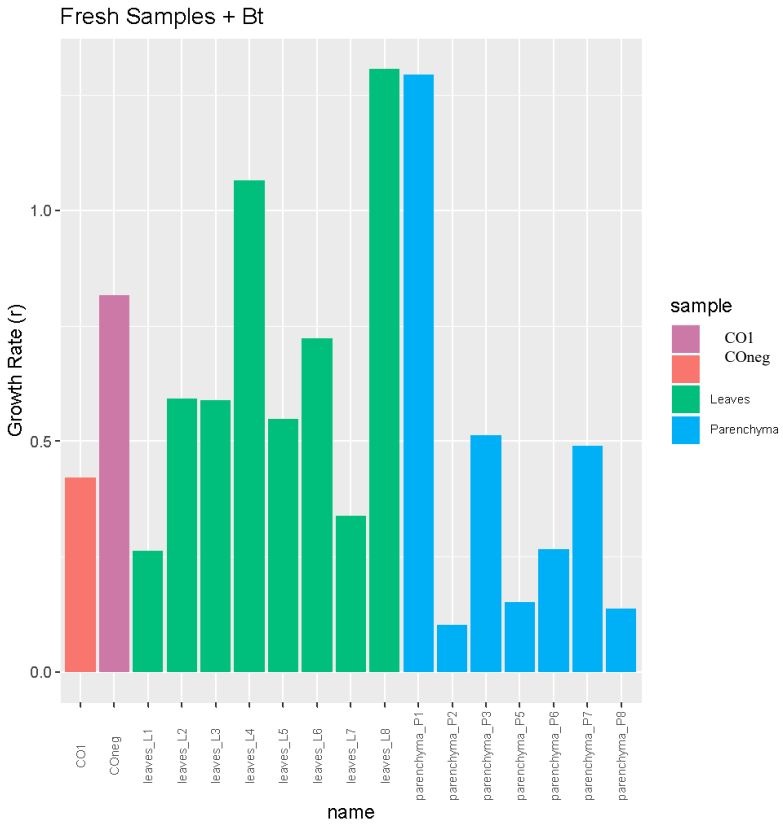
The same experiments were performed with fresh, living material. Here we could observe that the parenchyma extract can inhibit the growth of gram-positive bacteria (Fig. 4A/1). An inhibition with the extract from the leaves of the *Cecropia* plant could not be clearly observed, looking at the pooled data. Considering all samples separately it can be shown, that only single samples grew more compared to the controls (Cecropia leaves – 6:2; parenchyma – 7:1) (Fig.4A/2). Furthermore, the parenchyma samples, as well as the samples of the leaves showed no growth-inhibiting effect in *S. marcescens* (Fig. 4B/1 & Fig. 4B/2). Also, the ant samples did not inhibit the growth of *Bt*. or *Sm*. (Fig. 4C). Since neither the *Bt.* nor the *Sm.* control bacteria grew, it is not clear whether inhibition of bacterial growth took place in the ant sample (Fig. 4B/3). Of note, all the curves have high standard errors and are noisy, indicating that technical issues might have occurred.

A/1

Bt-


Fresh samples
Leaves, Parenchyma
Optical density (OD)
1.25
1.00
0.75
0.50
0.25
0
5
10
15
Time (hours)
treatment
CO1
COneg
leaves
parenchyma

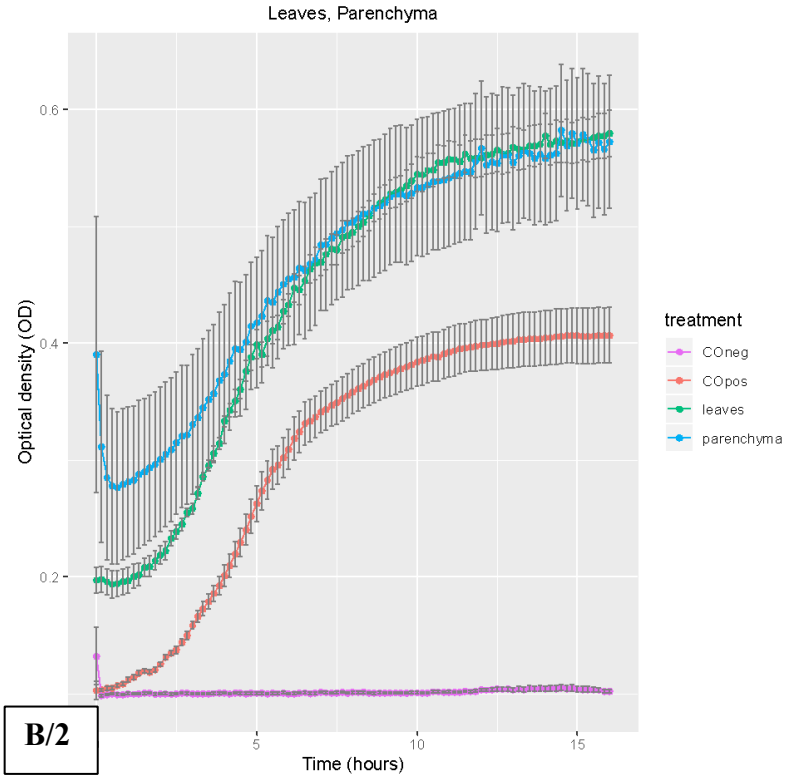
A/2


Fresh Samples + Bt
Growth Rate (r)
1.0
0.5
0.0
CO1
COneg
leaves_L1
leaves_L2
leaves_L3
leaves_L4
leaves_L5
leaves_L6
leaves_L7
leaves_L8
parenchyma_P1
parenchyma_P2
parenchyma_P3
parenchyma_P5
parenchyma_P6
parenchyma_P7
parenchyma_P8
name
sample
CO1
COneg
Leaves
Parenchyma

B/1

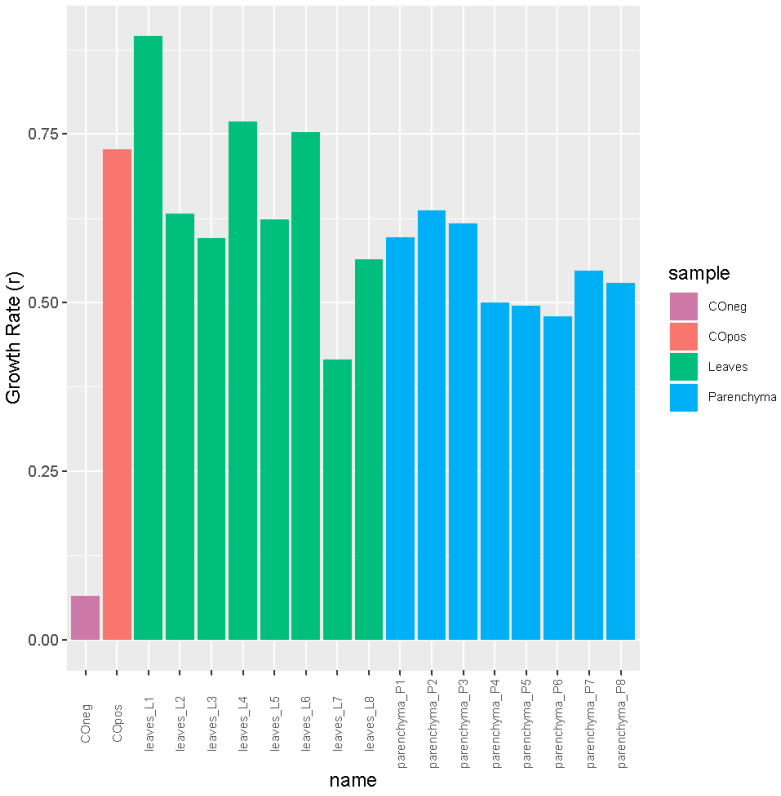
Sm-

Fresh samples



B/2

Fresh Samples + Sm



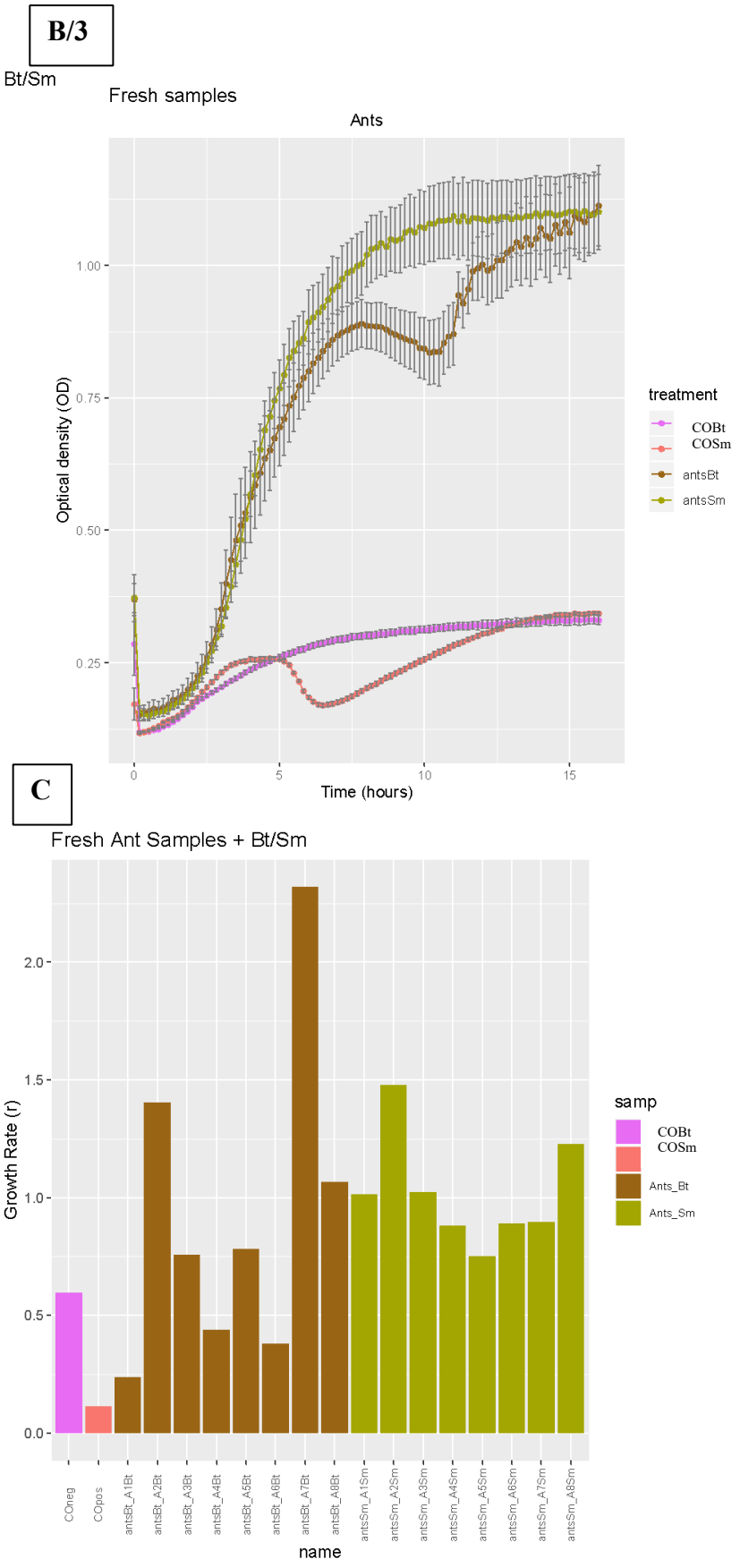


Figure 4. First panels: Mean and standard error for all biological replicates of fresh samples, *Cecropia* leaves, patch and *Azteca* ants. Second panels: Growth rate for the individual biological replicates. **(A)** Leaves and parenchyma extract incubated with *Bt*. **(B)** Leaf samples and parenchyma samples incubated with *Sm*. **(C)** This

graph shows the growth curves of *Bt*. and *Sm*. incubated with extracts of the ant samples. COBt, in this case only, contains 50µL of *Bt*. and 20µL PBS, while COSm is 50µL of *Sm*. and 20µL PBS.

5.2 Bacterial inhibition assay – LB agar plates

Since it was not possible to measure the optical density of the bacteria cultures with carton extracts (dried as well as fresh) or with extract of larvae, the sample extract-bacteria mixtures were applied to LB agar plates to assess the growth of the bacteria.

Interestingly, carton extract had an inhibitory effect on the growth of gram-positive *Bacillus thuringiensis*. By comparing the effect of carton extract of samples from three different *Azteca* colonies with the control plate (Fig. 5A-C), apparent differences between treatment and the control were observed (Fig. 5D). Only a few single colonies were observed here. An inhibition of gram-positive bacteria (*B. thuringiensis*) could be seen.

In contrast, no inhibitory effect on gram-negative *Serratia marcescens* was seen (Fig. 6A-C). Compared to the control (Fig. 6D), they were similarly overgrown. No clear inhibition of gram-negative bacteria was observed.

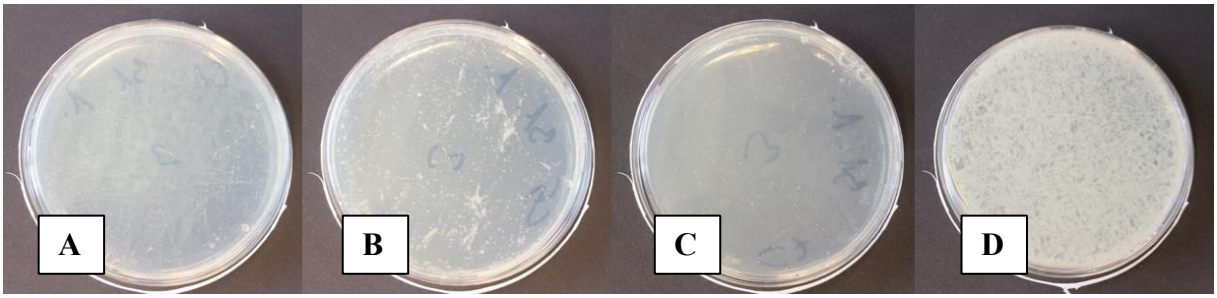


Figure 5. Growth of *Bt* with extract of three different carton samples (C2, C5, C7). There is a clear difference compared to the control plate **(D)** without carton extract.

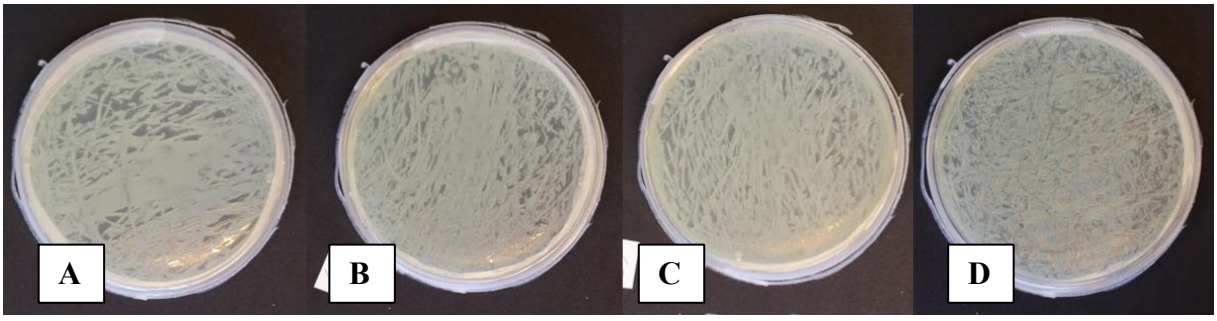


Figure 6. Growth of *Sm* with extract of three different carton samples (C2, C5, C7). There is no difference compared to the control plate **(D)** without carton extract.

The fresh carton samples showed similar growth-inhibiting effects on the gram-positive *Bt.* (Fig. 7A-C). Compared to the completely overgrown control plate (Fig. 7D). Similar to the dried material, there was no effect on bacterial growth of gram-negative *Serratia marcescens* (Fig. 8A-C). No visible differences could be observed between the experiments and the control. (Fig. 8D).

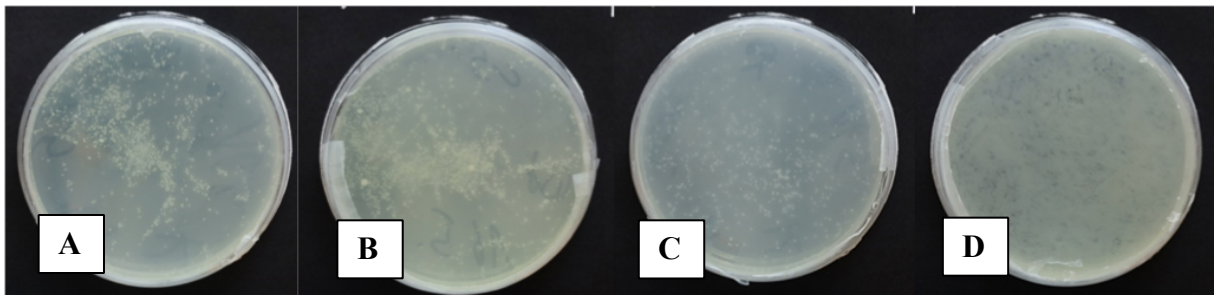


Figure 7. Growth of *Bt* with extract of three different carton samples (C1, C3, C5). There is a clear difference compared to the control plate **(D)** without carton extract.

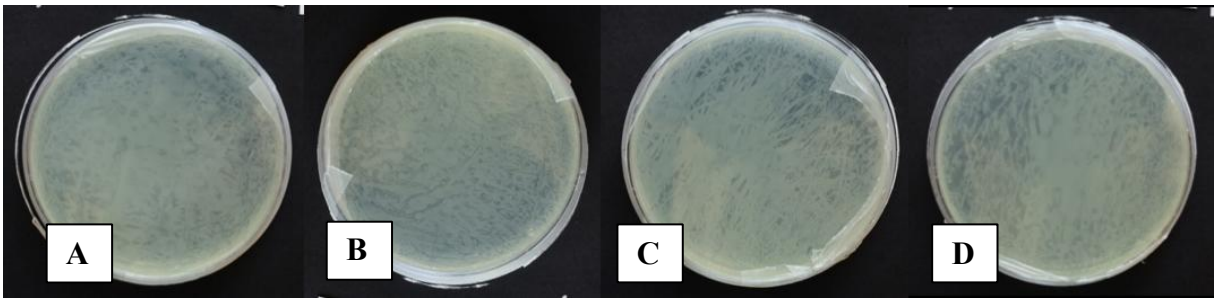


Figure 8. Growth of *Sm* with extract of three different carton samples (C1, C3, C5). There is no difference compared to the control plate **(D)** without carton extract.

No visible differences could be observed between the experiments and the control; neither for gram-positive Bt (Fig. 9), nor gram-negative Sm (Fig. 10). In comparison to the control, all the plates were similarly overgrown, both with *Bt*. (Fig 9A-D) and *Sm*. (Fig. 10 A-D).

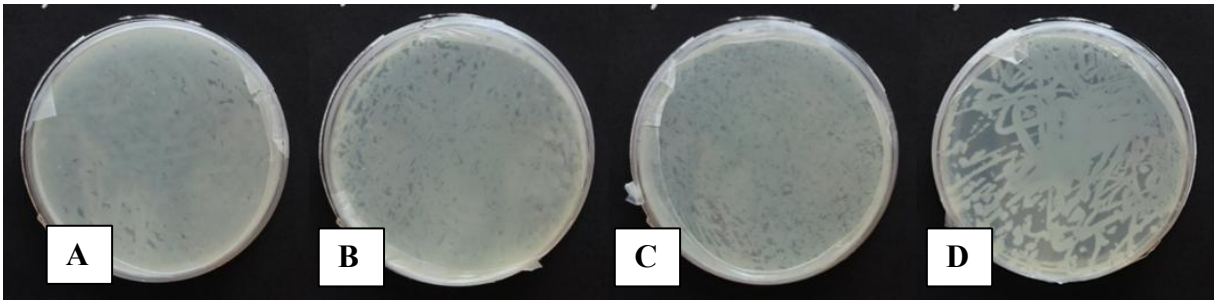


Figure 9. Growth of *Bt* with extract of three different brood samples (B3, B4, B7). There is no difference compared to the control plate **(D)** without brood extract.

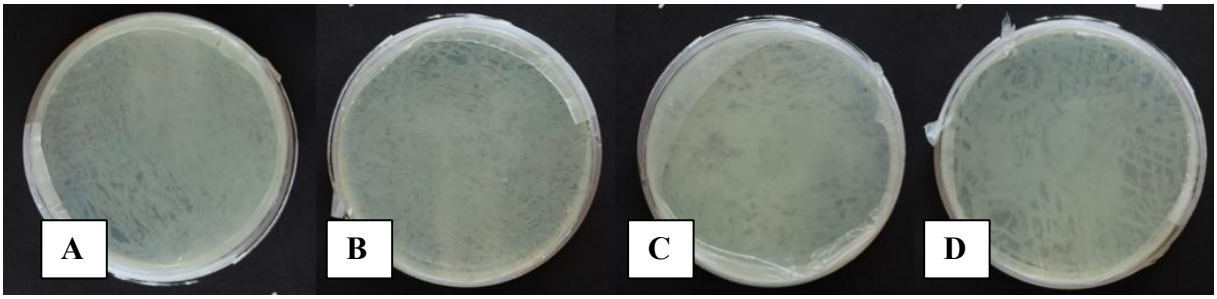


Figure 10. Growth of *Sm* with extract of three different brood samples (B3, B4, B7). There is no difference compared to the control plate **(D)** without brood extract.

5.3 Fungi – contact inhibition assay

In order to test the third hypothesis, whether the fungi have antifungal properties and helps to protect against fungal insect pathogens, the last experiment was performed.

Growing two isolates from the same fungus against each other, showed the most mycelium growth, i.e. no inhibition (Fig. 11A). When growing the ant associated fungus against the insect pathogenic fungus (*Metarhizium* sp.), the mycelium size seemed less pronounced than without *Metarhizium* (Fig. 11B). This would indicate that the ant fungus is able to mildly suppress the growth of the pathogenic fungus. It did not seem that *Metarhizium* could spread equally well and, indeed, it remained smaller in comparison to the *Metarhizium* only plates (Fig. 11C).

With the second fungus strand, similar results were achieved. Again, it could be observed that the ant fungus grew best against an isolate from the same fungus as its competitor (Fig. 12A). In competition with *Metarhizium*, one could see that the ant fungus as well as the insect pathogenic fungus, *Metarhizium*, did not grow as much as in the controls (Fig. 12B). The control for *Metarhizium* showed that the *Metarhizium* samples on those plates, spreaded the most and had nearly overgrown the entire plate (Fig. 12C).

This experiment indicates that the insect pathogen fungus is somehow inhibited in its growth when it grows in competition with an ant associated fungus.

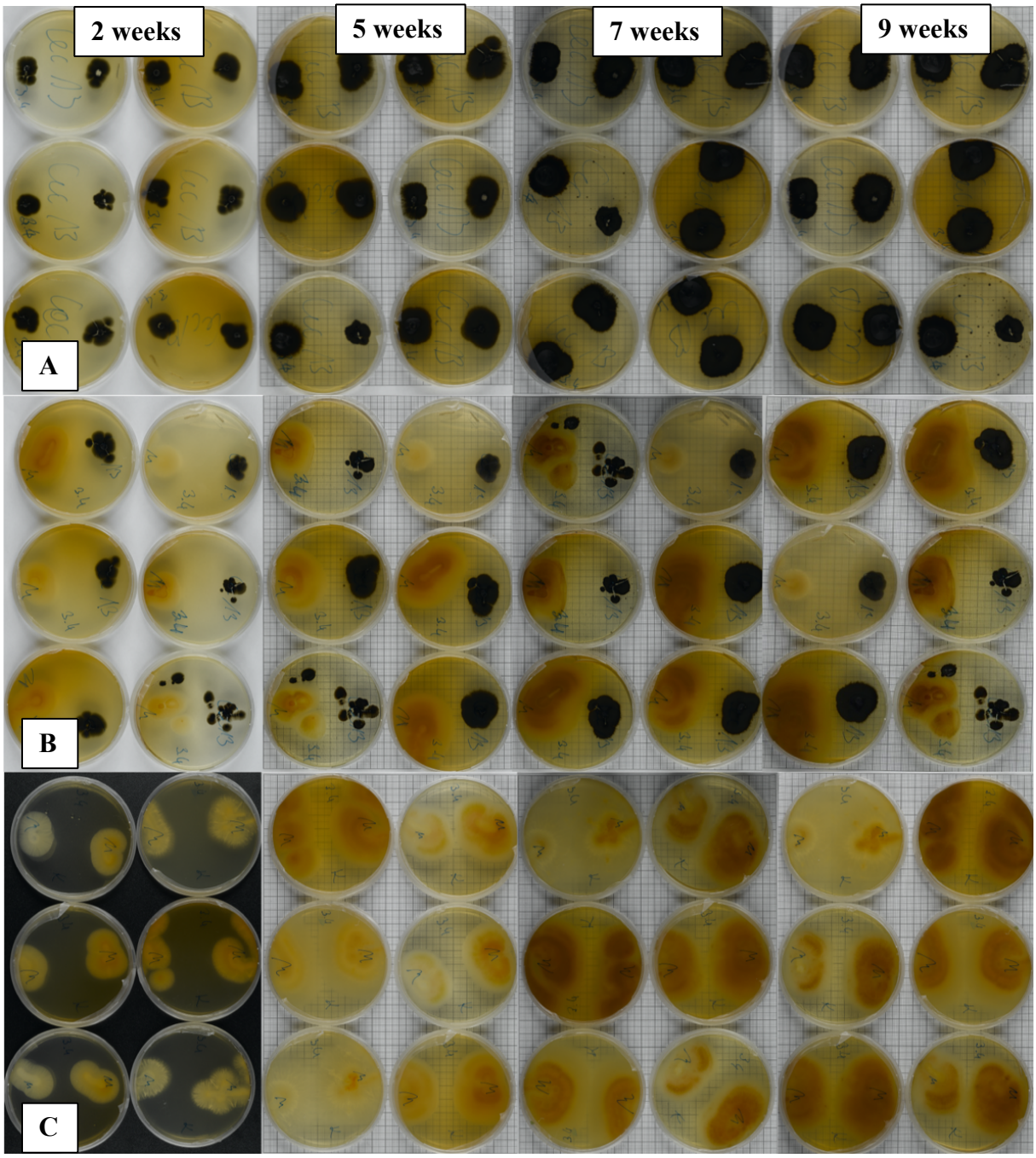


Figure 11. Fungi growth competition assays with a chaetothyrialean domatia fungus isolated from patches in an *Azteca-Cecropia* association (Cec13) and a strain of the insect pathogenic *Metarhizium sp.* A photographic overview over the observed growth period (9 weeks) of the fungi. **(A)** The first row shows the growth of the ant fungus strain (Cec13) against itself. **(B)** The second row shows the growth Cec13 and *Metarhizium sp.* as its competitor. **(C)** The last row shows the growth of *Metarhizium* against itself.

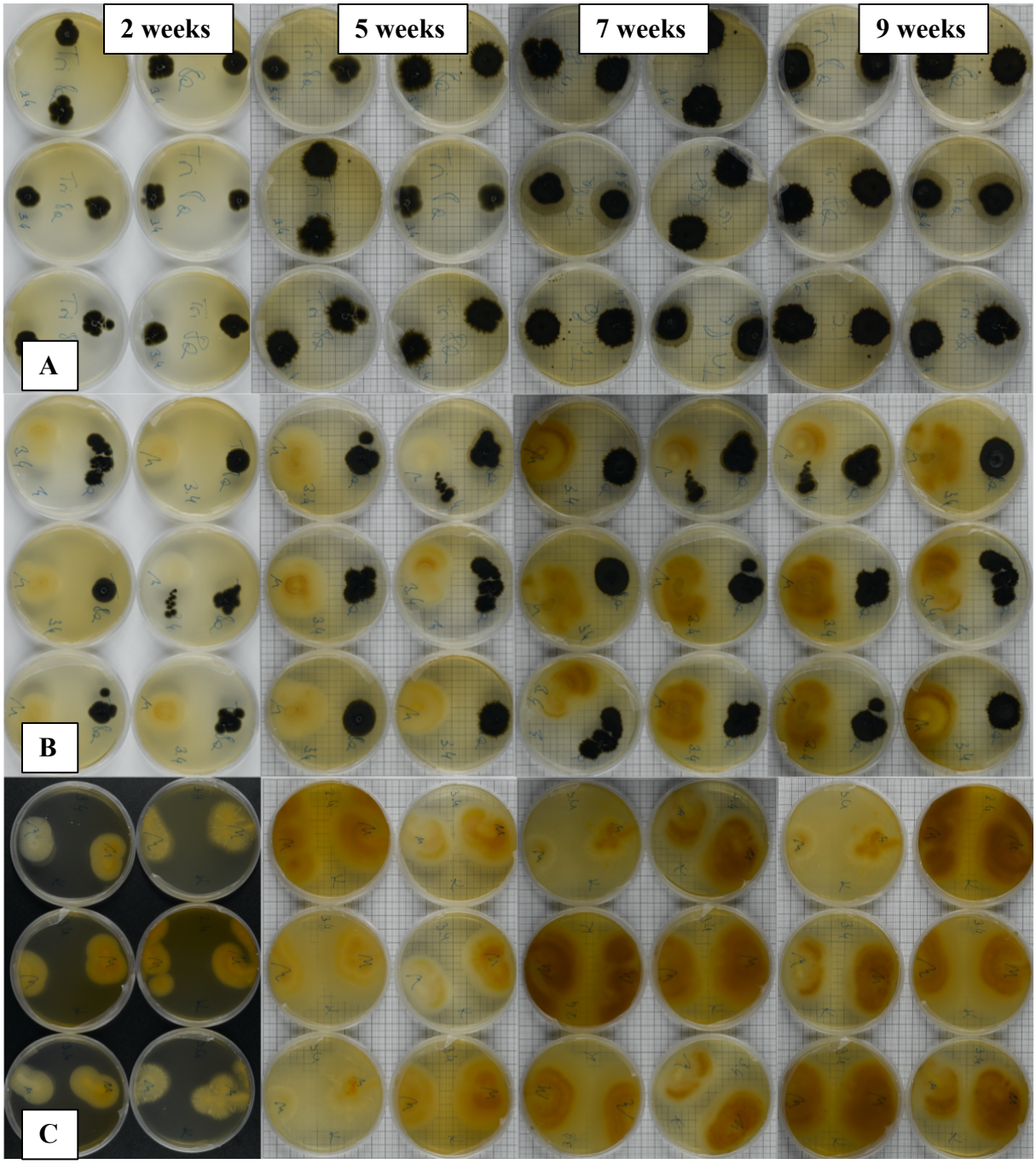


Figure 12. Fungi growth competition assays with a chaetothyrialean domatia fungus isolated from patches in a *Triplaris-Pseudomyrmex* association (Tri8a) and a strain of the insect pathogenic *Metarhizium sp.* **(A)** The first row shows the growth of the ant fungus isolate (Tri8a) against itself. **(B)** Timeline for the growth pattern of a Tri8a isolate with *Metarhizium sp.*, an insect pathogenic fungus, as its competitor. **(C)** The last row shows the growth of *Metarhizium* against itself.

6 Discussion/Conclusion/Outlook

It is known that *Azteca* ants living inside the hollow stems of *Cecropia* plants make small "compost" piles where they deposit waste, plant material and dead nestmates (Nepel et al., 2016; Mayer et al., 2018; Marting et al., 2018). Astonishingly, these compost piles are not in separate chambers and apart from the center of living as in some other ant-plant associations (e.g. myrmecophytic *Myrmecodia*, *Hydnophytum* or *Tococa* species) (Miehe, 1911; Huxley, 1978), inside the *Cecropia* stem the compost piles occur even next to the larvae. The purpose of this study was to better understand how *Azteca* ants keep their nests, especially the brood, clean and protected against microbial and fungal infections. The first hypothesis was that the ants produce substances that inhibit the growth of pathogenic bacteria and fungi to keep the colony, especially their brood, clean. The second hypothesis was to find out whether the host plant produces antimicrobial substances. For this purpose, extracts were made from total ants as well as from plant material and incubated with two insect pathogenic bacteria, *Bacillus thuringiensis* (gram-positive) or *Serratia marcescens* (gram-negative) both insect pathogenic bacteria

The experiments show, that the leaves of the host plant and the parenchyma which covers the inner wall of the domatia were able to naturally inhibit bacterial growth of gram-positive *Bacillus thuringiensis* (Fig. 3A). Also, the carton made by the ants from parenchyma almost completely suppressed *Bt*. growth. In contrast, none of the host plant samples had any effect on the growth of gram-negative *Serratia marcescens* (Figs. 3B, 4C, 6, 8). These bacteria showed a normal growth rate, namely that of a log function.

Cecropia has often been described as a medicinal plant. It was reported to have medicinal properties effective against disease like malaria (Uchoa et al., 2010) or diabetes (Andrade-Cetto & Heinrich, 2005) for example. Others have also shown that the extract of the leaves have significant anti-inflammatory effects (Pérez-Guerrero et al., 2001). It is, therefore, not surprising that I could find an inhibitory effect on the growth of bacteria. It is, however, not clear why the effect was only on *Bacillus thuringiensis* but not on *Serratia marcescens*. It may be due to the considerably thicker cell wall of gram-positive bacteria (Beeby et al., 2013)

The agar plate experiments with the carton extract show that the gram-positive bacteria strain grows less with carton extract and we can therefore speculate that the carton contains growth inhibiting substances (Figs. 5A-C, 7A-C). A probable antimicrobial effect of carton may explain why these structures are always crowded with larvae and pupae. Carton may not only

serve as a shelter for the brood but may also help to protect the larvae and pupae against pathogens. As the carton is largely made from masticated host plant parenchyma and the parenchyma also showed suppression on growth of gram-negative *Bacillus thuringiensis*, it is suggested that the active ingredients come from the plant.

The last experiment, the fungal growth competition assays, indicates that fungi bred by the ants also play an important role in the cleanliness of the nest (Figs. 11, 12).

To answer the question of how *Azteca* ants, keep their nest clean, it can first be stated that workers have been observed to mechanically remove the waste from their nest. Fernández-Marín et al. (2006) identified a substance (myrmicacin; 3-hydroxydecanoic acid) found in the metapleural glands of leaf-cutting ants. Further tests have found that this substance has antimicrobial and antifungal activity and helps protect the leafcutter ants against pathogens. The fact that the ants have been observed spraying their brood with a secretion and rotate the larvae between their mandibles (Fig. 13) supports this hypothesis.

Figure 13. *Azteca* sp. ants caring their brood by rotating the larvae between their mandibles. Photo credit: Veronika Mayer.

Our results with the dried material also suggest that *Azteca* ants may be able to produce the same or a similar substance. Since our results from the tests with living ants (workers and larvae/brood), only partially support this, the question remains whether this ant species is able to produce this or a similar substance. Since the production of such highly complex substances is extremely cost-intensive for the insects, one could hypothesize that the ants use substances from other sources. One possibility could be that they only use the active ingredients of the plant or the fungi instead of producing it themselves. The experiments performed with the plant and fungal extracts, could in part support this hypothesis. In addition, it would be interesting to know whether antimicrobial substances can be found in other *Azteca* species that have not entered into a mutualism with *Cecropia* and house their colonies in other places.

Recently, studies on the composition of the microbiome in nests of *Azteca trigona* were published by (Lucas et al., 2017). This ant species builds their nets above ground from ant exudates and masticated plant fiber. Although it was hypothesized that the ant microbiome should be relatively similar to the soil, this assumption could not be confirmed in the course of the study. This result suggests that the ants are able to actively shape or influence the composition of nest associated microorganisms. Differences can also be detected between different colonies. Especially the concentration of various *Lactobacillus* species was different from colony to colony, which is probably due to the different nutrition of the colonies. Earlier this year, the same research group (Lucas et al., 2019) investigated the bacterial and fungal microbiota in functionally separate chambers inside and outside nests of *Azteca alfari* in *Cecropia peltata* trees. They also found that different nesting sites (internal & external) had different microbial communities and the "nurseries" were generally less bacteria-rich. Suspected pathogens were actively suppressed in the chambers inhabited by the ants. Also, this study supports the theory that ants can affect their microbial communities in the nests and thus prevent accumulation of pathogens. It became clear that both, the bacterial and fungal communities in the nest of *Azteca* ants differ from those of the environment. Another finding that supports earlier research that ants have the ability to monitor, influence and cultivate microbiota in their nests. In addition, this work suggests that *Azteca* ants are able to limit the accumulation of fungal groups, if these could be harmful to the colony.

Leafcutter ants, for example, have developed a different strategy to deal with detrimental bacteria and fungi. They have symbionts on their integument, which produce antibacterial and antifungal agents and are clearly recognizable as "white turf" on the underside of the ants (Currie et al., 1999). However, no “white turf” is visible in *Azteca* species, making it highly unlikely that they also have similar symbionts.

In the last experiment, the fungi growth experiment, we could show that the insect pathogen fungus is inhibited in its growth when it grows in competition with the ant fungus. This indicates that the fungus bred by the ants is also potentially involved in the control of pathogenic fungi (Figs. 11, 12). Possibly, the fungus also produces a substance which partially restricts the growth of non-colony fungi. Members of the genus of *Escovopsis* for example produce candicins (Haeder et al., 2009). Whether this is also the case for the fungi used during our experiment is unclear, but unlikely, as this group appears to be specific in inhibiting members of the genus *Escovopsis* (Haeder et al., 2009).

There is relatively little known about the mutualism between *Azteca* and *Cecropia*, and there are still many open questions. It would be interesting to identify, and isolate which substance could be produced by the plant. For this, one could chemically analyze the leaves of the plant and the carton samples. For example, methods such as NMR, mass spectroscopy, or chromatographic techniques (HPLC / GC) could be used. In the future, it will be important to test the inhibitory effect of both the carton and the leave samples with the same method, in order to be able to systematically compare these.

Furthermore, it would certainly be exciting, to take samples from the mandibular and metapleural glands of the ants and to examine them individually. One could use the protocol by (Ortius-Lechner et al., 2000) and analyze compounds by gas chromatography and mass spectrometry. This method can be used to compare the contents of metapleural glands and venom glands of other *Azteca* species to find out whether the species that live in *Cecropia* mutualism have stopped producing antimicrobial substances as they can rely on the substances produced by the plant and thereby save the production costs.

Also, one should further investigate the role of the fungus in the system. Here, it would be interesting to use chemical analysis methods, like column chromatography, as well. One could also test the fungi against bacteria to verify if the fungus can produce antibacterial substances similar to the penicillin-producing fungi.

During my research I was able to gain valuable insights into this extremely fascinating system. Unfortunately, due to the restricted availability of material, the tests could not be repeated often enough to produce conclusive results. In the near future, it will be necessary to increase the number of samples tested, to not only confirm the present findings, but also to reduce the observed biological variability. Technical issues further limited our ability to quantify the efficacy and strength of the antimicrobial activity of the ants and themselves. Also, the fungal growth on the agar plates should be quantified systematically. Additional tests will also allow us to perform statistical analyses, with an increased number of replicates. However, we could show that the current experimental set-up is able to measure fungal and bacterial growth differences and show growth inhibition.

In conclusion, the present work paves the way for future experiments to hopefully unravel the individual components of the ant nest with respect to antimicrobial and antifungal activity. Especially in the age of antibiotic resistance, every source should be investigated, which can help in the discovery and research of new drugs.

7 References

Agrawal, A. A., & Dubin-Thaler, B. J. (1999). *Induced responses to herbivory in the neotropical ant-plant association between Azteca ants and Cecropia trees: Response of ants to potential inducing cues.* Behavioral Ecology and Sociobiology, *45*(1), 47–54.

Andrade-Cetto, A., & Heinrich, M. (2005). *Mexican plants with hypoglycaemic effect used in the treatment of diabetes.* Journal of Ethnopharmacology, *99*(3), 325–348.

Beeby, M., Gumbart, J. C., Roux, B., Jensen G.J. (2013). *Architecture and assembly of the Gram-positive cell wall.* Molecular microbiology 88(4): 664-672.

Bischof, S., Umhang, M., Eicke, S., Streb, S., Qi, W., Zeeman, S. C., April, N. (2013). *Cecropia peltata Accumulates Starch or Soluble Glycogen by Differentially Regulating Starch Biosynthetic Genes by Differentially Regulating Starch Biosynthetic Genes5 Cecropia peltata Accumulates Starch or Soluble Glycogen*. The Plant Cell, Vol. 25, No. 4, pp. 1400-1415

Blatrix, R., Djiéto-Lordon, C., Mondolot, L., la Fisca, P., Voglmayr, H., & Mckey, D. (2012). *Plant-ants use symbiotic fungi as a food source: New insight into the nutritional ecology of ant-plant interactions.* Proceedings of the Royal Society B: Biological Sciences, *279*(1744), 3940–3947.

Currie, C. R., Scottt, J. A., Summerbell, R. C., & Malloch, D. (1999). *Fungus-growing ants use antibiotic-producing bacteria to control garden parasites.* Nature, *398*(6729), 701–704.

Defossez, E., Selosse, M. A., Dubois, M. P., Mondolot, L., Faccio, A., Djieto-Lordon, C., Blatrix, R. (2009). *Ant-plants and fungi: A new threeway symbiosis.* New Phytologist, *182*(4), 942–949.

Fernández-Marín, H., Zimmerman, J. K., Rehner, S. A., & Wcislo, W. T. (2006). *Active use of the metapleural glands by ants in controlling fungal infection.* Proceedings of the Royal Society B: Biological Sciences, 273(1594), 1689–1695.

Gutiérrez-valencia, J., Chomicki, G., & Renner, S. S. (2017). *Molecular Phylogenetics and Evolution Recurrent breakdowns of mutualisms with ants in the neotropical ant-plant genus Cecropia (Urticaceae).* Molecular Phylogenetics and Evolution, *111*, 196–205.

Haeder, S., Wirth, R., Herz, H., & Spiteller, D. (2009). *Candicidin-producing Streptomyces support leaf-cutting ants to protect their fungus garden against the pathogenic fungus Escovopsis.* Proceedings of the National Academy of Sciences.

Huxley, CR. (1978). *The ant-plants Myrmecodia and Hydnophytum (Rubiaceae) and the*

relationships between their morphology, ant occupants, physiology, and ecology. New Phytologist 80: 231-268.

Konrad, M., Vyleta, M. L., Theis, F. J., Stock, M., Tragust, S., Klatt, M., Cremer, S. (2012). *Social transfer of pathogenic fungus promotes active immunisation in ant colonies.* PLoS Biology, 10(4).

Lucas, J., Bill, B., Stevenson, B., & Kaspari, M. (2017). *The microbiome of the ant-built home: The microbial communities of a tropical arboreal ant and its nest.* Ecosphere, 8(2).

Lucas, J. M., Madden, A. A., Penick, C. A., Epps, M. J., Marting, P. R., Stevens, J. L., Meineke, E. K. (2019). *Azteca ants maintain unique microbiomes across functionally distinct nest chambers.* Proceedings of the Royal Society B: Biological Sciences, 286(1908).

Marting, P. R., Kallman, N. M., Wcislo, W. T., & Pratt, S. C. (2018). *Ant-plant sociometry in the Azteca- Cecropia mutualism*. Scientific Reports. (November), 1–15.

Mayer, V. E., Nepel, M., Blatrix, R., Oberhauser, F. B., Voglmayr, H., & Fiedler, K. (2018). *Transmission of fungal partners to incipient Cecropia -tree ant colonies*. PLoS ONE 1–18.

Miehe, H. (1911) *Javanische Studien II. Untersuchungen über die Javanische Myrmecodia*. Abhandl. Math. Phys. Cl. K. Sachs. Gesell. Wiss. 32: 312-361.

Milutinović, B., Höfling, C., Futo, M., Scharsack, J. P., & Kurtz, J. (2015). *Infection of Tribolium castaneum with Bacillus thuringiensis: Quantification of bacterial replication within cadavers, transmission via cannibalism, and inhibition of spore germination.* Applied and Environmental Microbiology, *81*(23), 8135–8144.

Nepel, M., Voglmayr, H., Blatrix, R., Longino, J.T., Fiedler, K., Schonenberger, J., Mayer V.E. (2016*) Ant-cultivated Chaetothyriales in hollow stems of myrmecophytic Cecropia sp. trees e diversity and patterns.* Fungal Ecology. Vol 23 (131-140)

Nepel, M., Voglmayr, H., Schönenberger, J., & Mayer, V. E. (2014). *High diversity and low specificity of chaetothyrialean fungi in carton galleries in a neotropical ant-plant association.* PLoS ONE*, 9*(11), 1–10.

Ortius-Lechner, D., Maile, R., David Morgan, E., & Boomsma, J. J. (2000). *Metapleural gland secretion of the leaf-cutter ant Acromyrmex octospinosus: New compounds and their functional significance. Journal of Chemical Ecology, 26*(7), 1667–1683.

Pérez-Guerrero, C., Herrera, M. D., Ortiz, R., Alvarez de Sotomayor, M., & Fernández, M. A. (2001). *A pharmacological study of Cecropia obtusifolia Bertol aqueous extract.* Journal

of Ethnopharmacology, *76*(3), 279–284.

Roberts, D. W., & St. Leger, R. J. (2004). *Metarhizium spp., cosmopolitan insect-pathogenic fungi: Mycological aspects.* Advances in Applied Microbiology, *54*, 1–70.

Sagers, C. L., Ginger, S. M., & Evans, R. D. (2000). *Carbon and nitrogen isotopes trace nutrient exchange in an ant-plant mutualism.* Oecologia, *123*(4), 582–586.

Schupp, E. W., & Schupp, E. W. (1986). *International Association for Ecology Azteca Protection of Cecropia : Ant Occupation Benefits Juvenile Trees Oecologia Azteca protection of Cecropia : ant occupation benefits juvenile trees*. Oecologia, *70*(3), 379–385.

Sprouffske, K., & Wagner, A. (2016). *Growthcurver: An R package for obtaining interpretable metrics from microbial growth curves.* BMC Bioinformatics, *17*(1), 17–20.

Steinwender, B. M., Enkerli, J., Widmer, F., Eilenberg, J., Thorup-Kristensen, K., & Meyling, N. V. (2014). *Molecular diversity of the entomopathogenic fungal Metarhizium community within an agroecosystem.* Journal of Invertebrate Pathology, *123*, 6–12.

Tragust, S., Ugelvig, L. V., Chapuisat, M., Heinze, J., & Cremer, S. (2013). *Pupal cocoons affect sanitary brood care and limit fungal infections in ant colonies*. BMC Evolutionary Biology, 13(1).

Tranter, C., Graystock, P., Shaw, C., Lopes, J. F. S., & Hughes, W. O. H. (2014). *Sanitizing the fortress: Protection of ant brood and nest material by worker antibiotics.* Behavioral Ecology and Sociobiology, *68*(3), 499–507.

Wickham, H. (2011). *Ggplot2.* Wiley Interdisciplinary Reviews: Computational Statistics, *3*(2), 180–185.

Uchoa, Valdileia, et al. *"Antimalarial Activity of Compounds and Mixed Fractions of Cecropia Pachystachya."* Drug Development Research, vol. 71, no. 1, 2010, pp. 82–91.

8 Appendix

8.1 Material & Methods

PBS Buffer

unit size	item	composition
1000ml	**PBS 10x**	NaCl 80g/L
		KCl 2g/L
		Na_2HPO_4 14,4g/L
		KH_2PO_4 2,4g/L
		Millipore water

Media

unit size	item	composition
1000ml	**LB medium**	LB Broth powder 20g/L
500ml		Millipore water
250ml		
100ml		
20 plates	**LB Agar Plates**	LB Agar powder 35g/L
		Millipore water
		Petridishes
20 plates	**SDA standard agar**	Sabouraud dextrose agar 32,5g/L
		Millipore water
		Petridishes
500ml	**TritonX-100**	0,005L
		Millipore water

Sample description:

Silica gel dried material

Sample number	Sample ID	weight sample	Type	plant species	ant species
C1	18_L4	0,642	carton	*C. obtusifolia*	*A. constructor*
C2	18_74	1,102	carton	*C. obtusifolia*	*A. constructor*
C3	18_37	0,446	carton	*C. peltata*	*A. alfari*
C5	18_5	0,99	carton	*C. obtusifolia*	*A. alfari*
C6	18_1	1,31	carton	*C. obtusifolia*	*A. alfari*
C7	18_6	1,071	carton	*C. obtusifolia*	*A. alfari*
C8	18_7	1,207	carton	*C. obtusifolia*	*A. alfari*
P4	16_4	0,139	patch	*C. peltata*	*A. constructor*
P2	18_2	0,036	patch	*C. peltata*	*A. constructor*
P74	18_74	0,403	patch	*C. obtusifolia*	*A. constructor*
P24	16_24	0,068	patch	*C. peltata*	*A. alfari*
P9	16_9	0,316	patch	*C. obtusifolia*	*A. constructor*
A5	18_5	5 workers	ants	*C. obtusifolia*	*A. alfari*
A57	18_37	5 workers	ants	*C. peltata*	*A. alfari*
A54	18_54	5 workers	ants	*C. peltata*	*A. alfari*
A2	18_L2	5 workers	ants	*C. peltata*	*A. constructor*
A4	18_L4	5 workers	ants	*C. obtusifolia*	*A. constructor*
A74	18_74	5 workers	ants	*C. obtusifolia*	*A. constructor*
Cec13	cec13	2mL	Domatia fungus	*C. insignis*	*A. xanthochroa*
Tri8a	Tri8	2mL	Domatia fungus	*Triplaris melaenodendron*	*Pseudomyrmex* sp
L25	25	3,452g	Leaves	*C. peltata*	Feb2018, VM
L26	26	4,256g	Leaves	*C. peltata*	Feb2018, VM
L27	27	3,951g	Leaves	*C. peltata*	Feb2018, VM

L= leaves
P= patch
A= ants

Fresh material

Sample No	sample ID	weight sample	plant	plant height (cm)	domatia width (cm)	ant	location	Date	notes
L1/P1/A1/B1	19_15	50mg/50mg/30 workers/30 larvae	*C. peltata*	120	1,1	*A. alfari*	Finca amable	12.05.2019	
L2/P2/A2/B2	19_16	50mg/50mg/30 workers/30 larvae	*C. peltata*	210	2,4	*A. alfari*	Organic oil palm plantation Daniel Jenkin	12.05.2019	
L3/P3/A3/B3	19_17	50mg/50mg/30 workers/30 larvae	*C. peltata*	200	2,6	*A. alfari*	Organic oil palm plantation Daniel Jenkin	12.05.2019	very active ant colony
L4/P4/A4/B4	19_18	50mg/50mg/30 workers/30 larvae	*C. peltata*	300	4,4	*A. alfari*	roadside between Finca amable and Villa Briceno, opposite of banana plantation	12.05.2019	
L5/P5/A5/B5	19_19	50mg/50mg/30 workers/30 larvae	*C. peltata*	170	2,3	*A. alfari*	outside Daniel Jenkins plantation, facing the Rio Bonito	12.05.2019	very actice colony, many MKs
L6/P6/A6/B6	19_20	50mg/50mg/30 workers/30 larvae	*C. peltata*	250	4,3	*A. alfari*	same as 19_18	12.05.2019	
L7/P7/A7/B7	19_21	50mg/50mg/30 workers/30 larvae	*C. peltata*	200	2,2	*A. alfari*	border to Cachorros pasture	12.05.2019	upright bended plant small

L8/P8/A8/B8	19_22	50mg/50mg/30 workers/30 larvae	*C. peltata*	250	2,4	*A. alfari*	border to Cachorros pasture	12.05.2019	upright bended plant bigger

L= leaves
P= patch
A= ants
B= brood

9 Zusammenfassung

Der Mutualismus zwischen den Pflanzen der Gattung *Cecropia* und den *Azteca* Ameisen ist eine der am weitesten verbreiteten Symbiosen in neotropischen Ökosystemen. Die Pflanzen bieten den Ameisen Nistplätze in ihren hohlen Internodien, sowie glykogenreiche Nahrungskörper, von denen sich die Ameisen ernähren können. Als Gegenleistung für Unterkunft und Nahrung schützen *Azteca* Ameisen die Pflanze vor Fressfeinden und entfernen andere Pflanzentriebe, die versuchen die Wirtspflanze zu überwuchern.

Jüngste Studien haben gezeigt, dass die *Azteca* Ameisen regelmäßig sogenannte "patches" herstellen, die aus organischer Substanz (Parenchym, toten Nestkameraden und Fäkalien) bestehen. An diesen Stellen kultivieren sie melanisierte, langsam wachsende Pilze von der Gattung der *Chaetothyriales* (Ascomyzeten). Die Hyphen des Pilzes werden dann an die Larven verfüttert. Diese "patches" finden sich an vielen Stellen des Nestes und nachdem die Larven mit dem Pilz gefüttert werden, auch neben der Brut. Aufgrund der Bedingungen in den Regenwäldern Costa Ricas ist die Kolonie der ständigen Bedrohung durch Pilz- oder Bakterienpathogene ausgesetzt, die vor allem für die Brut eine besondere Bedrohung darstellen.

In der aktuellen Studie untersuchen wir, wie die Brut vor mikrobiellem Befall geschützt wird. Mit Hilfe von antimikrobiellen Tests wurde festgestellt, dass insbesondere die Blätter der Pflanze eine hemmende Wirkung auf das Wachstum von gram-positiven Bakterien, wie beispielsweise *Bacillus thuringiensis* (*Bt*.) haben. Es konnte allerdings keine hemmende Wirkung auf gram-negativen Bakterien, wie beispielsweise *Serratia marcescens*, beobachtet werden. Untersuchungen des Wachstums von *Bt*. auf LB-Agarplatten in Kombination mit Kartonproben haben gezeigt, dass auch dort eine Hemmung auftritt. Diese Beobachtung steht im Einklang mit der Tatsache, dass Karton aus zerkauten Pflanzenfasern besteht. Diese Masse wird von den Ameisen genutzt, um sogenannte Galerien, innerhalb des Pflanzenstammes, zu bauen. Auf ihnen wird auch die Brut abgelegt.

Zusammenfassend lässt sich festhalten, dass die vorliegende Arbeit, insbesondere im Zeitalter der Antibiotikaresistenz, den Weg für zukünftige Experimente ebnet, um die Rolle der einzelnen Bestandteile der *Azteca*-Kolonie in Bezug auf die antimikrobielle Aktivität zu untersuchen. Jede mögliche Quelle sollte untersucht werden, die zur Entdeckung neuer Medikamente beitragen kann.